STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

STAINING SOLUTIONS INCLUDED IN THE NATIONAL FORMULARY

Altho most users of stains do not realize it, recent editions of the National Formulary, published by the American Pharmaceutical Association, have contained formulae for staining solutions in a source that is available to druggists fairly generally thruout the United States. Unfortunately, however, no effort has been made in the past to keep these formulae up to date or to even make certain that they were entirely correct. This, of course, has been due to the fact that few members of the Pharmaceutical Association understand staining and very few biologists consult the National Formulary.

This situation is to be changed in the future. A representative of the American Pharmaceutical Association, Dr. Louis Gershenfeld, has been appointed on the Stain Commission and the chairman of the Commission has been made a member of the National Formulary committee. This arrangement results in full cooperation and two or three conferences have been held with the purpose of standardizing the staining formulae in the next edition of the N. F. and taking care to see that they agree with those recommended by the Stain Commission. It will be some time yet before this new edition of the Formulary is out[1] and still longer before it becomes official; eventually, however, this should result in reliable staining formulae being at the disposal of druggists anywhere in the country. This ought to make it possible for a physician requiring a small quantity of some staining solution to obtain what is needed from his druggist in comparatively reliable quality.

Another result which is to be expected from this cooperation between the Stain Commission and the National Formulary committee is a more official recognition of the former than it has ever had. The new edition of the Formulary will specify stains that have been certified by the Commission on Standardization of Biological Stains.

[1]The chapter on Materials and Preparations for Diagnostic Use is now out in preprint form, and may be obtained from the Amer. Pharamaceutical Assoc., Washington, D. C. Price $1.50.

Inasmuch as Federal and state legislation very generally specify National Formulary quality in connection with drugs and related products, this gives a semi-official standing to an organization whose specifications and approval are recognized in the Formulary.

It is felt, therefore, that this cooperation is likely to have advantages for users of stains in small quantities and that it will also add to the prestige of the Stain Commission.

—H. J. Conn.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the January number of this Journal.

Stains Certified Dec. 1, 1938 to Feb. 28, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Crystal violet	LC–14	94%	As histological, cytological, and bacteriological stain; also in bacteriological media	Dec. 7, 1938
Wright stain	NWr–12	....	As blood stain	Dec. 11, 1938
Alizarine Red S	NAr–2	....	As stain for bone	Dec. 14, 1938
Safranin O	NS–11	89%	As histological, cytological and bacteriological stain	Dec. 27, 1938
Carmine	CCa–5	....	As histological and cytological stain	Dec. 27, 1938
Hematoxylin	FH–14	....	As histological and cytological stain	Jan. 20, 1939
Eosin B	LEb–1	87%	As histological counterstain	Jan. 23, 1939
Methylene blue	CA–17	86%	As histological and bacteriological stain; and as constituent of blood stains	Feb. 3, 1939
Basic fuchsin	CF–17	92%	For general staining, Feulgen stain, and in bacteriological media	Feb. 8, 1939

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each due for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

DISSECTION AND PREPARATION OF WHOLE MOUNTS OF ENDOSPERM FROM THE SEEDS OF *GREVILLEA* (PROTEACEAE)

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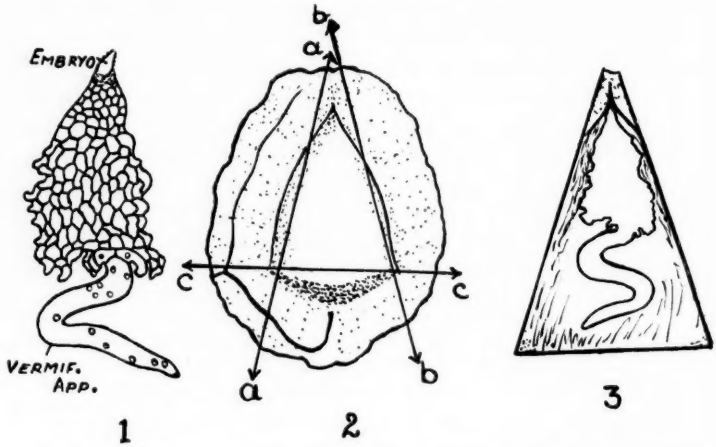
ABSTRACT.—**It is sometimes very desirable to supplement the examination of serially cut microtome sections by dissection and study of whole mounts of objects. In the case of the seeds of *Grevillea* the endosperm forms a curious worm-like structure, the *vermiform appendage*, in elucidating the nature of which microtome sections alone are inadequate. The dissection of the endosperm for demonstrating this structure is very necessary; otherwise the structure is altogether overlooked. In dissecting out the endosperm, which is easily done, either fresh seeds or previously preserved seeds may be used; the former are easier for handling and yield more satisfactory results. In the case of fresh material, an immediate killing is necessary and any one of the ordinary killing fluids may be used; Bouin's fluid was tried by the writer and was entirely satisfactory. After killing, the material is stained and dehydrated, the several stages of which are best done on the slide alone on which the material is placed. Mounting is done in any of the mounting media, and Canada balsam answers the purpose quite well.**

While it cannot be disputed that with the introduction of the microtome, great facilities are available to the modern plant morphologist for cutting uniformly thin serial sections for examination of minute details, the value of dissection for preparing whole mounts for study may sometimes become surprisingly significant. Recently, Buchholz[1] has demonstrated that in the case of conifer embryos serial microtome sections alone fail to reveal a complete and intelligible picture of the tortuous suspensor systems and has stressed the importance of dissecting out the embryos and preparing whole mounts by which the coiled suspensor can be easily followed and studied. The present note is intended to point out another instance where an examination of microtome sections needs to be supplemented by a study of whole mounts prepared by dissecting the endosperm from the seed in *Grevillea*, a plant belonging to the Proteaceae.

[1]Buchholz, J. T. 1938. Dissection, staining, and mounting of the embryos of conifers. Stain Techn. **13**, 53–64.

The endosperm in *Grevillea* shows an upper cellular and a lower cenocytic portion. The latter forms a highly coiled tubular structure, worm-like in appearance and called the *vermiform appendage* of the endosperm, which was for the first time discovered by the writer and is elsewhere fully described.[2] Serial microtome sections of the seed, even when cut fairly thick, are not entirely satisfactory nor adequate for determining the exact nature of this coiled structure. Such preparations merely show bits of the *appendage* distributed in a number of sections. A dissection of the endosperm, which can be mounted whole, is on the contrary very desirable to get a full understanding of not only the relations between the cellular portion and the non-cellular coiled structure, but also in determining exactly the true nature of the coiled structure itself. (Fig. 1).



Figs. 1 to 3. Dissection of Endosperm from the seeds of *Grevillea*. *Fig. 1:* A whole mount of endosperm dissected out of a seed of *Grevillea robusta* showing the *vermiform appendage*. ×14. *Fig. 2:* An entire seed with the broad wing; the lines indicate how the margins may be cut away by a scalpel. ×3. *Fig. 3:* One piece of the seed coat (seen from inside) after cutting away the margins of the seed as indicated above; the whole mass of endosperm with its *vermiform appendage* is seen loosely hanging from the micropyle and may be lifted up on the point of a mounted needle. ×12.

In preparing whole mounts of the endosperm, it is important to procure seeds at the right stage. The pericarp of the fruit containing such seeds is quite green in color and the embryo inside the seeds is small and consists of a spherical or oval mass of cells in which the differentiation into the histogenic layers has just become recognizable.

[2]Kausik, S. B. 1938. Studies in the Proteaceae. I. Cytology and floral morphology of *Grevillea robusta* Cunn. Ann. Bot. N.S., **2**, 899–910.

The seeds in *Grevillea* are quite flat and are, therefore, easily and securely placed on the slide for dissection. The margins of the seed are cut away as shown in Fig. 2 and the seed coat is then lifted off in two pieces. This process is easily accomplished as the tissue inside the seed coat is almost completely disorganized and a large cavity is formed. The mass of endosperm is freely suspended inside this cavity. After the seed coat is removed, this mass of endosperm usually lies loosely adhering to one of the two pieces (Fig. 3) and may be easily picked up on the point of a fine mounted needle and placed on a clean slide for further treatment and mounting.

The dissection of the endosperm may be performed either with fresh or with preserved seeds. The former are always preferable for dissection, whereas the preserved material shows the endosperm a little crushed and shrunken. After the mass of endosperm is completely removed and placed on the slide, the following procedure in killing, staining and mounting may be followed. At all stages in this procedure it is always best to retain the material on the slide alone, on or from which drops of various fluids may be added or withdrawn as necessary by means of an ordinary medicine dropper.

Killing and Staining

If the material is fresh and not previously preserved the mass of dissected out endosperm needs to be killed in any of the ordinary killing fluids. Bouin's fluid was used by the writer and the results obtained were entirely satisfactory. The killing is over in about an hour after which the material is washed in three or four changes of distilled water and then stained. If Heidenhain's iron-alum hematoxylin is used, as was done by the writer in most cases, a preliminary mordanting in 4% iron-alum is necessary, after which the material is again washed in changes of water and stained for about two hours. The material is then destained in 2% iron-alum, washed in water and dehydrated in grades of alcohol and finally brought up into pure xylene, the entire process being over in about an hour. The material is next ready for mounting which is quite satisfactorily done in clear and fairly thin Canada balsam.

In the case of material which was previously in some preservative, as for example in formalin-acetic-acid-alcohol, the material is first washed thoroly in several changes of 50% alcohol, brought down to water gradually and is then subjected to the same treatment as in the case of fresh material outlined above. As previously stated, however, it is preferable to have fresh material, as the results are more satisfactory.

Instead of hematoxylin, safranin or any other stain may also be used, either singly or in the usual combinations. In the case of safranin stained material it is always good to retain a little more stain than is actually required at the end, as the excess of stain is lost in passing the material thru the grades of alcohol while dehydrating. If Venetian turpentine is employed in place of Canada balsam, in mounting the material and sealing, unnecessary labor and careful watching are involved; Canada balsam answers the purpose well.

The dissection of endosperm for whole mounts was first successfully tried by the writer in *Grevillea robusta* and was subsequently followed in the case of a few other species of the Proteaceae, namely *Grevillea banksii*, *G. hilliana* and *Macadamia ternifolia*. In the first two the presence of the *vermiform appendage* of the endosperm was again noticed, while in *Macadamia*[3] its place and function were found to have been taken over by the formation of irregular multinucleate lobes of the lower region of the endosperm. Yet another species, *Hakea saligna*, was also examined, but the seeds that were available were rather old and the endosperm was completely used by the embryo in these. This will be taken up again at the earliest opportunity.

The earlier workers in the family, including Messeri,[4] who investigated *Grevillea macrostachya* and Brough,[5] who worked on *Grevillea robusta* have failed to demonstrate the existence of the *vermiform appendage* of the endosperm; and these, in all probability, confined themselves to an examination of microtome serial sections alone and thus overlooked an important feature of the endosperm. It is undoubtedly of great interest to find out if similar or corresponding features are present in the other members of the Proteaceae. It is the intention of the writer in contributing this brief article that interested readers, who may be able to secure seeds of any of the Protead genera, will look for this peculiar feature in the development of the endosperm.

[3]Kausik, S. B. 1938. Studies in the Proteaceae. II. Floral anatomy and morphology of *Macadamia ternifolia* F. Muell. Proc. Ind. Acad. Sc., **8**, 45–62.

[4]Messeri, A. 1928. Embriologia di *Grevillea macrostachya* Brongn. et Gris. Nuovo Giorn. Bot. Ital., Nuova serie, **34**, 1037–42.

[5]Brough, P. 1933. The life history of *Grevillea robusta* Cunn. Proc. Linn. Soc. N.S.W., **58**, 33–73.

COMPARISON OF METHODS FOR DEMONSTRATING GLYCOGEN MICROSCOPICALLY

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ABSTRACT.—**Three methods for the microscopic demonstration of glycogen are compared: namely, Best's carmine, the iodine method, and Bauer's modification of the Feulgen technic. The lability of glycogen necessitates immediate fixation, small pieces of tissue, and agitation of the fixative. The fixative recommended consists of 9 parts by volume of absolute ethyl alcohol to one part of 40% formaldehyde neutralized with $MgCO_3$; the alcohol in this formula may be saturated with picric acid if desired. After fixing, the tissue should be washed in absolute alcohol and embedded in paraffin or celloidin, taking care to avoid overheating of blocks or sections. A sharp knife should be used in sectioning. The Best's carmine technic is employed by staining first with hematoxylin, then for 20 minutes in: Best's stock solution (prepared by the usual formula, using Grübler's carminum rubrum optimum), 10 cc.; concentrated ammonia, 15 cc.; methyl alcohol, 30 cc.; used fresh after agitating and without filtering. The iodine method calls for Lugol's aqueous iodine. The Bauer-Feulgen method calls for sections mordanted 1 hour in 4% aqueous CrO_3 and washed in water 5 minutes, followed by 10-15 minutes in: a suitable basic fuchsin (e. g. Grübler), 1 g.; warm dist. water, 100 cc., with filtration of solution while still warm; normal HCl, 20 cc., added after cooling; $NaHSO_3$, 1 g.; ripened to a straw or amber color for 24 hours in the dark. After this bath the sections are rinsed 1½ minutes each in 3 changes of M/20 $NaHSO_3$, then 10 minutes in running water, and are then counterstained with hematoxylin. Of these three methods the iodine technic proves a good check on either of the others. Saliva digested controls are also essential, taking care to wash out mucus from sections.**

From time to time one sees in the literature statements as to the relative accuracy and dependability of the various methods for the detection of glycogen in microscopic sections. For many years we have been studying glycogen histologically and some results have been published. Numerous requests have been received for information relative to the fixing and staining of glycogen in tissues; and this seems to be an opportune time to place our experience at the disposal of others engaged in similar studies.

Before proceeding to a discussion of the staining methods, it is important to emphasize the fact that glycogen in fresh tissues is a

labile material. It may disappear rapidly from many tissues post mortum in part or completely. For this reason prompt fixation is essential; the tissue should be in the fixative in less than 10 minutes after the cessation of the blood flow. It is important to use small pieces of tissue (2–3 mm. thick) thru which the fixing fluid can rapidly penetrate.

While in the fixing fluid the tissue should be moved about frequently but gently. A piece of tissue lying in the bottom of a dish of strong alcohol remains in a dilute alcohol solution for hours unless agitated. The agitation also allows the fixing fluid to penetrate from all sides at once and so completes the penetration of the block in less time than otherwise.

We have found three fixatives reliable, that is, they always preserve a maximal amount of glycogen when used as we have indicated.

A—Absolute ethyl alcohol

B—Absolute alcohol.....9 parts (by volume)
Pure 40% formaldehyde solution neutralized with $MgCO_3$...1 part

C—Absolute alcohol saturated with picric acid.....9 parts
Neutral formaldehyde solution.....1 part (Lison 1936)

The sections should be cut with a sharp knife and mounted very flat and smooth. This is particularly important for Best's carmine (see p. 49).

Four staining methods are of special value.

Best's Carmine

The stock solution is prepared as follows:

Grübler's carminum rubrum optimum	2 g.
Potassium carbonate	1 g.
Potassium chloride	5 g.
Distilled H_2O	60 cc.

Boil gently until the color darkens. Allow to cool. Add 20 cc. concentrated ammonia. Ripen 24 hours.

In making up this formula it is important to select a carmine that will stain glycogen; as some will not. It is possible, of course, by chemical treatment to modify a carmine that is unsuitable for this formula so as to make it work, but since most biologists are not familiar enough with the chemical processes involved, it saves time to select a carmine that is satisfactory and use it exclusively. After trying a large number, some satisfactory, some not, Grübler's carminum rubrum optimum has proved uniformly satisfactory. For use the solution is prepared as follows:

To 10 cc. of the stock solution add 15 cc. of concentrated ammonia and 30 cc. of pure methyl alcohol. Stir thoroly and use at once. *Do not filter.*

This will stain the glycogen a brilliant vermilion-red color, sometimes with a metallic gleam. It is used as follows:

The sections, cut in paraffin and fastened to slides with albumin, are brought down to water, then stained with a hematoxylin solution suitable for a nuclear stain. We use R. R. Bensley's modification of Ehrlich's hematoxylin.

They are then put in the freshly diluted Best's carmine solution for 20 minutes. If glycogen is present this is enough time and does not give too much chance for the background to become pink, if that tendency is present. When testing a new tissue for glycogen it is well to run a section of properly fixed liver or the like along with the first lot of sections stained in a given batch of stain to make sure that the stain is working properly.

The sections are then rinsed in about three changes of pure methyl alcohol. In the first washing a good bit of precipitate comes off the slides, so this alcohol should not be used again. They may now be cleared and mounted in the ordinary way with absolute alcohol and xylene, but we prefer to use acetone for dehydration and toluol for clearing.

In spite of precautions indicated above, one of the difficulties to contend with in the use of Best's carmine is the frequent adherence of precipitate to the sections. One soon, however, becomes fairly proficient in distinguishing this from glycogen. Another trouble is a tendency of the sections to develop a pink tinge all over, even in the nuclei which tho previously well stained in hematoxylin may change color in the carmine. Lacking a sharp color contrast between the various cell constituents, material of this type is most difficult to study even tho vermilion-red glycogen is present, and in these the salivary digestion controls help very little. So far we have not been able to do much about this difficulty. Apparently it is due to something inherent in the material or impurities in the fixing or dehydrating reagents and not to faulty staining solutions. It has occurred with various types of tissue supposedly promptly fixed, and a specimen that gives this result will do so even when sections from the same block are obtained years apart. Here, too, prompt fixation of the tissue may make a difference.

If glycogen in a specimen fails to stain with Best's carmine, pay more attention to the freshness of the solutions. One cannot tell how long such a colloidal solution will keep but the stock should remain satisfactory for some weeks and the dilute solution at least a few days. It seems preferable to make a fresh solution of the latter each time it is needed. Also, solutions from older stock will some-

times stain only part of the glycogen present and this may be a paler shade. Such solutions gradually lose their glycogen staining capacity entirely.

The Iodine Method

We use this method for the most part as recommended by Gage, but with some modifications for ease and accuracy of use, with our particular material.

Iodine colors glycogen a reddish-brown if it can reach it, but during the section mounting process factors may appear which keep the tissue from taking up the iodine, even when there is plenty of glycogen present.

Mount the sections in the usual way, being very careful not to heat them more than absolutely necessary to remove wrinkles, dry, remove paraffin with xylene or benzol, transfer thru alcohols to water, leave 10 or 15 minutes in a Lugol's aqueous iodine solution, and allow to dry in the air after blotting with filter paper. They must be protected from dust and not left too long before mounting or they will fade again. When they are dry, mount in yellow vaseline, as recommended by Gage, being very careful not to heat them too much.

Drying the sections does perhaps distort the cells somewhat, and air bubbles from the dried section work out into the mounting medium, but after a day or two they are relatively clear, and the glycogen results are uniformly good. Any preparations made in this way are not generally so good for high-power study as those made with Best's carmine or with the Bauer-Feulgen method, but nevertheless it is a useful control for other methods.

Gage recommended two methods of using iodine for glycogen. One may use either aqueous or alcoholic Lugol instead of water for spreading the paraffin sections. If this is done it is as important not to overheat the sections as it is when using the method recommended above, else they may not give the reaction with either aqueous or alcoholic iodine. For more permanent preparations, place the dried paraffin sections directly into alcoholic Lugol without removing the paraffin, dry, remove paraffin with xylene, and mount in yellow vaseline. This method, in my experience, is apt to give very variable color reactions in different sections of a series mounted on a single slide even when care has been taken not to overheat any part of the slide.

The Bauer-Feulgen Method

This test consists in the application to glycogen of the Riche and Bardy reaction for aldehydes as modified by Schiff and later by Feulgen. It consists in freeing aldehydic groups by a moderate

hydrolysis and then detecting them with a solution of basic fuchsin decolorized by sulfurous acid. A good discussion of it as applied to glycogen may be found in Lison (1936, p. 231). It may be called the Bauer-Feulgen test for glycogen as it is actually the Feulgen method which Bauer modified for specifically staining glycogen.

The Feulgen reagent is made as follows:

Dissolve 1 g. of basic fuchsin[1] in 100 cc. dist. water by heat. Filter warm and add to the filtrate, when cool, 20 cc. of normal HCl. Add 1 g. of $NaHSO_3$. Allow the solution to stand 24 hr. before using, when it should be a pale straw color.

Sections cut in paraffin are mounted on slides in the usual way, put in 4% chromic acid for 1 hour, or 1% chromic acid overnight. Wash in running water for 5 minutes. Place in the Feulgen reagent for 10–15 minutes. Then rinse in 3 changes of a molecular solution of $NaHSO_3$ one part, tap water 19 parts. The sections should remain 1½ minutes in each change. Wash in running water for 10 minutes.

One may now clear and mount the sections, or the latter may be first counterstained with a good nuclear stain. For this purpose Ehrlich's hematoxylin (Bensley, 1938) has been used with good success in this laboratory.

If the technic is successful, glycogen should be stained a deep red color with a violet tinge, and if the hematoxylin has been used for counterstain the nuclei will be a clear lavender color.

In both the Best's carmine and Bauer-Feulgen methods, particularly in the latter, it is advisable to agitate the slides frequently in all solutions to insure even distribution of color in different sections on the slide. An extended study shows that a failure to do so may result in considerable color difference between adjacent serial sections.

Saliva Digestion

Since none of the three methods described stains glycogen exclusively, no study of the distribution of glycogen in tissues is complete without the saliva digestion control.

Each time a new lot of material is stained with either the Best's carmine or Bauer-Feulgen methods, some sections should first be treated with saliva, then stained along with the others for comparison.

After the slides have been brought down to distilled water, cover the sections with saliva (spit on them) and allow them to stand from 15 minutes to ½ hour, changing the saliva several times. By this time all glycogen will be changed into sugar. Then wash very thoroly in water of about body temperature, as any mucus remaining will take up the stain and cause trouble later on. When counter-

[1]Grübler product used by the writer.

stained one can see at once where glycogen has been removed and whether or not other pink or red-stained materials are present.

Comparison of Methods

It requires exceptional care and considerable experience to make fully reliable glycogen preparations. If the particular glycogen in a tissue is especially labile and it is important to demonstrate all the glycogen present at a given time, then precautions must be taken to fix it immediately and uniformly in strong alcoholic solution (see p. 48). If plasmolysis of the intracellular glycogen is to be reduced to a minimum, fixation in aqueous formol-chrom-sublimate (Zenker-Bensley) or a similar one is indicated, but the chances are then against all the glycogen being preserved. If there are chrom-salts in the fixative and intracellular glycogen is to be studied, then the Best's carmine method must be used for staining because the iodine method is unsatisfactory for high-power work and the Bauer-Feulgen method does not stain glycogen specifically under these conditions. In alcohol fixed material all three methods are equally reliable. The Bauer-Feulgen method gives sharper microscopic pictures, small glycogen granules are easier to see, and the magenta color always distinguishes it from pink or red stained mucins or the like.

It is well to remember that these are staining methods and not microchemical tests and before we can speak with certainty as to the glycogen content of a tissue, it is necessary to make a chemical determination of glycogen. Where accurate quantitative data are needed, such determinations are essential.

Conclusion: There are, of course, advantages and disadvantages to the Best's carmine, Iodine and Bauer-Feulgen methods, and while one may become fairly expert at distinguishing glycogen from other cell constituents, it is never safe to judge a material of unknown glycogen content without some, and preferably all the controls possible, and to use several of the methods for comparison with each other. If one can fix one's own material promptly, however, in alcohol or formol-alcohol, the Bauer method is by all odds the one to use for beauty of color contrast, facility of study and accuracy of interpretation.

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THE USE OF JANSSENS' IRON HEMATOXYLIN IN PLACE OF THE WEIGERT ACID IRON CHLORIDE HEMATOXYLIN

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Some time ago the junior author, in search for a more satisfactory iron hematoxylin stain for tissue cultures, tried the old Janssens'[1] formula for an iron hematoxylin which Janssens called "hématoxyline noire," and which he described as having given "dans certaines circonstances des colorations d'une électivité tout à fait remarquable." It was found that this solution stained in 4 or 5 minutes, did not overstain in 30 minutes, and that a sample 4 weeks old was quite satisfactory. In this study the amount of ferric ammonium sulfate in the solution was reduced, from a saturated solution (in excess of 120 g. per 100 cc.) as recommended by Janssens, to 40 g. per 100 cc.

As we were using an iron hematoxylin and picro-fuchsin as a routine stain in the pathology laboratory we decided to give this iron hematoxylin formula a more extended trial. First a 160 cc. lot of Weigert's hydrochloric acid iron chloride hematoxylin, the formula previously in routine use, was made up and a record kept of the number of slides stained. This was used from June 18 thru June 25, and a total of 444 slides were stained. Those stained on the last day showed weakening of the nuclear staining and the solution was no longer considered satisfactory. Our usual experience, as well as that of others, has been that the Weigert formula is good for 7 or 8 days only.

We then prepared on June 20 a batch of 320 cc. of Janssens' formula from the same lot of hematoxylin but with the amount of alum further reduced, as follows:—

2 g. hematoxylin (Biosol, lot No. 84) was dissolved in 60 cc. absolute methyl alcohol (neutral, acetone free). This was added to a solution of 20 g. ferric ammonium sulfate (iron alum, violet crystals) dissolved in 200 cc. distilled water. To the whole, 60 cc. of C. P. glycerin were added.

The mixture was a deep blue-violet when freshly prepared and stained more intensely than when used later. In about an hour it

[1]Janssens, F. A. and Leblanc, A. 1898. Recherches cytologiques sur la cellule de levure. La Cellule, **14**, 203–9. (Year incorrectly given as 1897 in Lee's Microtomists Vade Mecum, 9 ed., p. 154, and 10 ed., p. 155.)

assumed a brownish black color and thereafter its staining properties were quite constant. Half of this solution was placed in the Stender staining jar, and to compensate for evaporation and carry-over two replenishments of 80 cc. each were made on July 5 and 13. The stain was used almost daily for 5 weeks, during which time a total of 1175 slides were stained. It was discarded July 25 as the nuclear stain showed some weakening.

Janssens' iron hematoxylin is considered, therefore, to be stable for 4 or 5 weeks, as compared with 1 week for Weigert's; it gives indistinguishable staining from *fresh* Weigert's iron hematoxylin, stains satisfactorily in 5 minutes, does not overstain in 30 minutes, and when the greater stability of the solution is considered, consumes less hematoxylin.

THE PREPARATION OF HARDENED EMBEDDING PARAFFINS HAVING LOW MELTING POINTS

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ABSTRACT.—**Technical "stearic acid" hardens paraffins melting at 52° C. and above, and at the same time lowers the melting point. Spermaceti wax further lowers the melting point of such a mixture without much effect on the hardness. With these two substances, and one of the anti-crystallizing adjuvants already found satisfactory, embedding media yielding thin sections at room temperature and having melting point below 52° C. can easily be prepared. A general method is given, specific formulas are stated, and the behavior at various temperatures of typical embedding media of this kind is described.**

For the safe infiltration of delicate material, the embedding paraffin should have a melting point not higher than 52° C., and for the best results in cutting 3 μ and thinner sections from material embedded in an ordinary embedding paraffin having such a melting point, the room temperature must be from 0° to –2° C. (Chamberlain, 1932). Otherwise, apparatus and tissue must be chilled with ice. An embedding paraffin having the required low melting point, but hard enough at the normal room temperature to yield thin sections without cooling devices of any kind, would obviously be much more convenient. If the cutting of thin sections at normal laboratory temperatures could be made possible, it also seemed that the cutting temperature of the paraffin block would be more nearly uniform, stable, and reproducible. Furthermore, ribbons of such a hardened paraffin would not become limp and sticky after leaving the knife, since they would not have to be brought into contact with an atmosphere of a temperature higher than that at which they were cut. The writer has, therefore, studied the properties of numerous waxes and other substances with a view to modifying the relationship of the melting points of paraffins to their room temperature hardness.

The best results, thus far, have been obtained with *technical "stearic acid"* (the "stearin" of commercial candle-stock mixtures), supplemented by smaller additions of *spermaceti wax*. With use of these two readily available modifiers and a suitable anti-crystallizing adjuvant it became possible to prepare from the common commercial

[1]3218 Stephenson Place, N.W., Washington, D. C.

paraffins embedding media melting at temperatures below 52° C. and hard enough at a temperature between 23° and 24° C. (74° F.) to permit the cutting of good 3 μ and fairly good 2 μ sections. At about 71° F., 1 μ sections were cut (Table 2).

Technical "stearic acid" (stearic acid containing palmitic acid and small percentages of other fatty acids) did not, at first, seem very promising for use in embedding media because it has a high melting point (55°–60° C.) and must be used in rather high percentages. As a matter of fact, however, stearic acid does not raise the melting point of an ordinary paraffin but actually *lowers* it (probably because the two substances are not of an isomorphous crystalline structure) to an extent depending upon the grade of stearic acid used and increasing with the percentage added up to more than 25%. Geller (1935) reproduces part of a melting-point table prepared by Lüdecke (1926, p. 108) to show the effect of from 2–25% of various waxes and other substances on the melting point of a paraffin, melting in the pure state at 52° C. According to these data, 14% of a stearic acid preparation melting at 55° C. lowered the m. p. of this paraffin to 50° C., and the 25% mixture had a 49.2° C. m. p. In the writer's experiments with the "triple-pressed" grade, from 16–20% of technical stearic acid hardened very effectively, and the larger percentages reduced the m. p. of the common commercial paraffins (m. p. about 54°–55° C.) to about 52°. Further lowering by stearic acid alone, however, could only be had by carrying the hardening to such a point that only the thinner sections (5 μ or less) could be cut at normal room temperatures without excessive curling. A lower m. p. with less extreme hardening can be had by starting with a slightly softer paraffin base. In the writer's work on these mixtures, however, a better modification in this direction has thus far been obtained by including a small proportion of spermaceti wax with the stearic acid.

Spermaceti wax (m. p. 42°–47° C.) was suggested by the late G. L. Bidwell[2] as another possible hardener, as it is harder than paraffin and has so low a m. p. that it should lower the m. p. of the mixture. This wax proved, however, to have the more valuable property of lowering still further the m. p. of paraffins to which stearic acid had been added, without appreciable effect in either direction upon the hardness conferred by the stearic acid. The hardness may, therefore, be adjusted, by means of the stearic acid, to suit both the expected laboratory temperature (winter or summer) and the range of section thickness desired; and, within certain limits, any further reduction

[2]Food Control Division, U. S. Dept. Agric., Food and Drug Administration, Washington, D. C.

of m. p. which the nature of the paraffin base may require can then be secured by means of a suitable percentage of spermaceti. The limiting proportion of this wax seems to be about 7%. Larger percentages began to lessen the cohesiveness and tenacity of the paraffin. On the other hand, as little as 1% of spermaceti increased distinctly the rate, relative to the percentage of stearic acid added, at which the latter modifier lowered the m. p. of the paraffin.

Anti-crystallizing adjuvants have already been proposed by several investigators. Their effects do not seem to be appreciably modified by the presence of technical stearic acid and spermaceti. Johnson (1903) added 1% of crude rubber, with a trace of asphalt. Keeping the paraffin melted for a long time, however, caused a part of the rubber to precipitate. McClung (1929) gives Johnson's formula, and adds that small quantities of castor oil may be effective. Chamberlain (1932) finds that bayberry "wax" (a vegetable tallow, or hard fat, not a true wax) "is likely to improve any paraffin except the best." The quantity specified would amount, by weight, to about 0.1%. The same author notes that Land found asphalt very effective. Waddington and Kriebel (1935) added 0.5% of a "petroleum ceresin," more fully described by Higgs (1935) with special reference to the modification of industrial paraffins. This proportion (0.5%) of "petroleum ceresin" slightly *raised* the m. p. of the paraffin. Higgs, however, found as little as 0.3% sufficient to produce the desired "microcrystalline" condition in paraffins, and this smaller proportion did not affect the setting point. It should be noted that "petroleum ceresin" is quite distinct from the ceresin prepared by refining ozokerite, and also from any form of paraffin.

To this list the writer can add Japan "wax" (like bayberry wax, it is actually a vegetable tallow), which has an effect practically indistinguishable from that of bayberry wax; the chloroform-soluble components of hard coal tar (suggested by Land's use of asphalt) when freed from solvent by prolonged heating; and bleached ozokerite or white ceresin.[3] Of the tar preparation, as little as 0.05% was effective, and did not change the m. p. Like asphaltum, however, it caused a marked dull yellow coloration. The ozokerite preparation, m. p. 72° C., checked crystallization at a concentration of 0.1%. At 0.5% it slightly raised the m. p.

The paraffin base: The cutting properties of the unmodified paraffin seem to have little or no influence on those of the embedding medium

[3]The writer is indebted to Dr. George S. Jamieson, Bureau of Chemistry and Soils, U. S. Dept. Agric. for the samples of ozokerite ceresin and candelilla wax used in the experiments described, and for much information on waxes, vegetable tallows, and related substances.

obtained by combining it with the adjuvants here considered. The common commercial paraffins, tho no one of them is a good embedding medium *per se*, have given quite as good results in the preparation of stearic-spermaceti paraffins as have the paraffins sold especially for biological work. Paraffins from the western "crudes" are said to have higher melting points than have those from the eastern oils.[4] The writer found the m. p. of "Texwax" more than 1° C. higher than that of "NJ Parowax." The latter paraffin thus far has given slightly better results in stearic-spermaceti mixtures than has any other paraffin base, either a biological or a commercial paraffin.

Soft paraffins (m. p. about 45° C.) are not suitable for hardening with stearic acid. Their melting points are not lowered but raised by the hardener, and good cutting properties are not produced. A minor proportion of such a soft paraffin added to a hard paraffin (m. p. 50°–55° C.) may, for some purposes, give better results than the hard paraffin alone. (See formula SSB 2, described below.)

The preparation of stearic-spermaceti paraffins: Except for occasional attention to funnel temperature during filtration, the preparation of the modified paraffins requires but a few minutes. The paraffin base is melted and held at a temperature of from 60°–70° C., the stearic acid ("triple-pressed" grade) and spermaceti wax are added, together with from 1–2% of either bayberry or Japan wax (tallow). These components are mixed thoroly and the completed embedding medium is poured into a hardened filter paper in a hot-water-jacketted funnel. In a funnel large enough to take 0.5 Kg. of the paraffin at once this quantity passes thru a hardened paper in about 40 minutes.

The thin grayish crust found on the surface of some lumps of bayberry wax does not dissolve in melted paraffin and is difficult to filter out. This coating should be completely removed before the lump is cut for weighing. Japan wax causes a slight opalescence which does not filter out. In the writer's experiments thus far, however, this has been so faint as to be harmless.

Formulas and behavior of some typical SSB (stearic-spermaceti-bayberry) paraffins: The paraffins described below have all given good results at the temperature and section thickness specified. Their formulae should, however, be regarded as illustrative only. The general method is obviously adaptable, by alteration in the percentage of the hardener, both to higher or lower cutting temperatures and to other section thickness.

Melting points were determined by heating very slowly 3 g. samples, in which the thermometer bulb was immersed, until the en-

[4]Information furnished by Dr. G. S. Jamieson.

tire sample melted to a clear, thin liquid. Smaller samples gave results lower than those observed in the paraffin oven. Cutting tests were made on a rotary microtome. The blocks were cut 1 cm. square; the block, microtome, and knife being brought to room temperature before each test. To secure absolutely uniform conditions of comparison, the data of Table 2 were obtained from blocks of the same size and without any embedded material. These paraffins have also been tested practically in the cutting of similar sections of *Vicia faba* root tips.

The formulae of the following paraffins are shown in Table 1.

TABLE 1. COMPOSITION OF STEARIC-SPERMACETI PARAFFINS 1–4.

Formula Number	Techn. Stearic Acid	Sperma-ceti Wax	Bayberry "Wax"	Paraffin Base	Nature of Paraffin Base
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	
SSB 1	16.	3.	1.	80.	"NJ Parowax"
SSB 2	16.	3.	1.	80.	70% "NJ Parowax"+30% soft paraffin (45° m.p.)
SSB 3	25.	1.	1.	73.	"Texwax"
SSB 4	25.	1.	1.	73.	"NJ Parowax"

Paraffin SSB 1 melted to a clear, thin liquid at 51.3° c., which was 3.2° below the m. p. of the pure paraffin from which it was made. By operating the microtome slowly, smooth 7 μ sections were cut at 86° F. At 83° F., with slow cutting, the 3 μ sections were fairly good, the 4 μ sections good, and the 5 μ sections very good. These 5 μ sections expanded much less than 10% on the slide (cf. Chamberlain's criteria, footnote under Table 2) and showed only a slight permanent compression. At 74° F., equally good 3 μ sections (*Vicia faba* root tips) were cut. At 69° F. the 3 μ sections were still more crisp, were slightly bowed unless the microtome was run rather briskly, and broke apart readily when long ribbons were cut. For further data on 3 μ and 2 μ sections, see Table 2. Satisfactory 1 μ sections were not obtainable from SSB 1 at normal room temperatures.

Paraffin SSB 2 was made from a paraffin base (m. p. 51.9° C.) consisting of a 3:7 mixture of 45° C. paraffin with "NJ Parowax." The m. p. of this embedding medium (49.6° C.), was the lowest thus far obtained in a paraffin yielding thin sections at normal temperatures. The behavior of this paraffin at 69° F. is shown in Table 2. At 79° F. it yielded (at a very slow cutting rate) good 5 μ and 4 μ sections, and fair 3 μ sections. The 2 μ sections were poor at this temperature, however. For the infiltration of material easily injured by heat SSB 2 is the best of the formulae here given, but it obviously does not stand the test of high summer temperatures as well as does SSB 1.

Paraffins SSB 3 and SSB 4 were made up to determine if the hardness could be raised to a point at which 1 μ sections could be cut at ordinary room temperatures without loss of other essential properties. "Texwax" has a melting point (55.6° C. in the case of the samples tested by the writer) higher than that of "NJ Parowax" (54.5° C.), and it therefore seemed quite possible that the "Texwax" preparation (SSB 3) might be harder than that made from "NJ Parowax" (SSB 4). The reverse was the case, as is shown in Table 2. Both yielded 1 μ sections, but the paraffin base of the lower m. p. gave in this case an SSB preparation of definitely better hardness.

TABLE 2. BAHAVIOR OF CERTAIN SSB PARAFFINS AFTER STANDING 18 HOURS IN A TEMPERATURE-CONTROLLED ROOM AT FROM 69°–71° F.*

Referance No. of paraffin	Melting point °C.	Cutting temperature °F.	Section thickness	Expansion on warming†	Fraction of full width recovered
SSB 1	51.3° C.	71° F.	3 μ	3.5%	0.92
SSB 1	51.3° C.	71° F.	2 μ	4.2%	0.84
SSB 2	49.6° C.	69° F.	3 μ	5.3%	0.85
SSB 2	49.6° C.	69° F.	2 μ	5.9%	0.80
SSB 3	51.0° C.	71° F.	1 μ	12. %	0.65
SSB 4	50.6° C.	71° F.	1 μ	11. %	0.71

*The wrinkling and permanent compression data here shown refer to sections cu at a rate much slower than is customary in the use of a rotary microtome. Except in the handling of long ribbons, a good sliding microtome might be more suitable for the cutting rate needed for the 2 μ and 1 μ sections.

†Compare Chamberlain's criteria (1932, p. 126) for 5 μ and 1 μ sections: "If a 5 μ ribbon does not lengthen when warmed on the flooded slide more than 5%, the cutting may be regarded as good; at 1 μ, if it does not lengthen more than 20% the cutting has been good."

Advantages and Disadvantages: The convenience of the hardened paraffins is obvious. The desirability of an approximately reproducible cutting temperature has already been noted, together with the probability of better behavior of the hardened ribbons in warm air. To test the heat tolerance of such ribbons, about 20 cm. of a ribbon 3 μ thick (SSB 1) was left on dry paper thru two days of warm weather (maximum about 88° F.). At the end of this time the entire ribbon could still be picked up intact.

A test of one of these hardened paraffins was made by Dr. M. N. Pope[5] who found, as disadvantages of this type of embedding medium, that the blocks are not as transparent as are those of a pure paraffin; and that great care had to be used in flattening the ribbons on the

[5]Of the Bur. of Plant Industry, U. S. Dept. of Agriculture, Washington, D. C. The writer is also indebted to Dr. Pope for the opportunity to use a Spencer rotary microtome in obtaining the data given in Table 2; and for the suggestion to measure and include in this table the data given in the last column.

slide, since too much warmth set free the sections from the paraffin. Advantages noted by Dr. Pope were that slower solidification occurs than in the case of pure paraffins; that there was little deformation; and that the ribbon handled well at 89°–90° F., sticking neither to the instruments nor to the fingers at this temperature.

Like all hard paraffins, the stearic-spermaceti hardened media do not ribbon readily when the temperature is a little lower than that best suited to the thickness of the sections. A hot needle, dipped into soft paraffin, may be applied to the upper and lower surfaces of the block (Chamberlain, 1932, p. 125). The writer, however, prefers the heated end of a slide. A single touch with the slide covers the whole surface with a thin and even coat of the softer paraffin. In using the hardened paraffins here described, this treatment may well be given every block as soon as it has been mounted on its wooden support. This will avoid an unknown change of temperature after the block has come to room temperature and is ready for cutting. For the same purpose—that the room temperature may correctly represent the actual cutting temperature—the writer avoids touching either the paraffin or the knife with his fingers in attaching the paraffin block to the microtome. If these two precuations are taken, the degree of hardening best adapted to the prevailing laboratory temperature and the required section thickness can easily be determined and thereafter adhered to.

The *solubilities* of the stearic-spermaceti paraffins seem to be essentially the same as those of pure paraffins. This statement applies specifically to solubility in the benzene hydrocarbons; in chloroform and carbon tetrachloride; in the butyl alcohols; and in *p*-dioxan. The stearic-spermaceti paraffins show much less tendency to separate in long, acicular crystals from saturated solutions in benzene, toluene, or xylene than do the pure paraffins. A previous article has already described (Waterman, 1934, p. 28) how the writer has utilized the re-solution, on warming, of suspensions of paraffin in the butyl alcohols to secure a gradual increase in the concentration of the paraffin solution to which the material is exposed before oven infiltration. The difficulty met with by McWhorter and Weier (1936) in securing complete infiltration after dioxan has been encountered by the writer after tertiary butyl alcohol and after dioxan. This was overcome by following the McWhorter and Weier method, consisting in the addition of a little xylene in two steps preceding oven infiltration.

Higgs' (1935) observations on the effect of small quantities of "petroleum ceresin" on paraffins would seem to make it almost certain

that this substance will prove superior to either bayberry or Japan wax if a commercial source of "petroleum ceresin" can be found.

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A NILE BLUE CULTURE MEDIUM FOR LIPOLYTIC MICROORGANISMS[1]

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ABSTRACT.—**A culture medium for the enumeration of lipolytic microörganisms is presented which makes use of Nile blue sulfate as a specific indicator for detecting changes in neutral fats. It is prepared as follows:—20 g. agar (Bacto) is dissolved in 1000 cc. beef infusion broth prepared in the usual way; the whole filtered and the reaction adjusted to pH 7.4; 100 cc. amounts are distributed in flasks and sterilized in the autoclave at 15 pounds pressure for 20 minutes. A dye-fat emulsion is then prepared by first dissolving 4 g. ossein gelatin (200 Bloom, isoelectric point at pH 5.6) in 100 cc. distilled water at 60° C. and sufficient N/10 NaOH added to bring the reaction to pH 7.2. The gelatin solution is first shaken vigorously in a stoppered bottle which contains 100 cc. U.S.P. cotton-seed oil to which has been added 0.1 g. Nile blue sulfate (C.I. No. 913, dye content 90%, solubility 0.15 g. in 100 cc. water) and then put thru a hand homogenizer until the oil globules are about 10μ in diameter. After sterilization in the autoclave at 15 pounds pressure for 20 minutes, the emulsion which was originally blue, turns deep pink.**

When plates are to be poured, 100 cc. of the sterile infusion agar base are liquified and while still hot, 10 cc. of the sterile dye-fat emulsion are added aseptically. The whole is well mixed and allowed to cool to 45° C. before being poured into plates. Before inoculation the medium has a rich salmon-pink color. Colonies of organisms which decompose fats appear as light blue colonies surrounded by a narrow zone of decolorized, transparent medium when incubated for 3-4 days at room temperature. Non-lipolytic colonies appear white or pink. The medium is equally efficient both with pour and steak plates, the former being desirable for quantitative work.

INTRODUCTION

During an investigation[2] undertaken for the purpose of establishing

[1]The author wishes to thank Dr. Louis Gershenfeld, Director of the Bacteriological Laboratories of the Philadelphia College of Pharmacy and Science, for the criticisms offered by him during the course of this investigation.

[2]Details of investigation submitted as thesis for the degree of Doctor of Science in Bacteriology at the Philadelphia College of Pharmacy and Science.

a relationship between the chemical changes that occur during the anaerobic decomposition of sewage sludge and the various microörganisms present therein, the necessity arose for making quantitative determinations of organisms possessing lipoclastic properties. After many unsuccessful attempts had been made to prepare and utilize several media which had been advocated for this purpose, the culture medium reported here was formulated.

Historical

Since Nencki's (1886) classic work on microbial lipolysis in the intestinal tract, a variety of culture media have been suggested for demonstrating the presence of fat-splitting microörganisms. In most instances, the proposed media employed one of the following for detecting changes in the fat: (a) indicators such as litmus and brom phenol blue for noting changes in acidity, Buchanan (1921), Waksman and Daviston (1926), Sayer, Rahn, and Farrand (1908) and Jensen and Grettie (1933); (b) production of colored soaps thru the use of copper sulfate and other metallic salts uniting with free fatty acids, Carnot and Mauban (1918) and Berry (1933); or, (c) specific indicators such as Nile blue sulfate, for free fatty acids, Turner (1929) and Smith (1907).

Jensen and Grettie (1937) made a comprehensive study of those media which employed the above and tho unable to find one entirely satisfactory, they reported that Turner's medium in which Nile blue sulfate was used, was superior to all other media investigated by them. Other evidence of the superiority of Nile blue sulfate as a specific indicator for detecting lipoclastic changes may be found in the work of Hammer and Collins (1934).

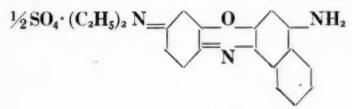
Considerable controversy has arisen regarding the kind of fat which should be utilized in preparing the fat emulsion employed in the medium. Turner (1929) employed cotton-seed oil. Jensen and Grettie (1937) who recommended and used cocoanut oil thought that cotton-seed oil, because of its high active oxygen content, inhibited the growth of many microörganisms. On the other hand, Hammer and Collins (1934) state that butter fat, beef tallow, cotton-seed oil and many other natural fats may be used with equally satisfactory results.

The media of Turner, of Jensen and Grettie, and of Hammer and Collins, were formulated by the respective authors primarily for quantitative purposes. Attempts by the writer to prepare these media satisfactorily were unsuccessful. This was due to the fact that sufficient data concerning the source and quality of the ingredi-

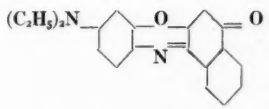
ents, especially of the Nile blue sulfate and the method for preparing the fat emulsion, were not given in the original publications.

Action of Nile Blue Sulfate on Fat

According to Conn (1936), Nile blue sulfate is a dye of the quinone-imine group. Its property of differential staining was first observed by Smith (1907) in studies on the staining of pathological sections. He observed that certain dyes of the oxazine series colored fat globules red while the protein matter was stained blue. Thorpe (1907) investigated this phenomenon and finally concluded that only dyes which are derivatives of phenonaphthoxazine yielded a red coloring matter that possessed a marked affinity for neutral fats. He also found that those derivatives containing an amino or substituted amino radical in position 6 exhibited this property to a greater degree. According to Thorpe, the action of water or very dilute mineral acid on Nile blue sulfate causes hydrolysis of the amino group in position 6 resulting in the formation of the corresponding oxazone as shown below.



Oxazine



Oxazone

The extent to which this hydrolysis takes place depends upon the temperature and the length of time during which the acid is allowed to act; but a condition of equilibrium between the salts of the two bases appears to be reached after boiling with water or dilute acid for 15 minutes.

The salts of both the oxazine and oxazone bases are blue and there is consequently no apparent change in the color of the dye solution after hydrolysis. The salt of the weaker oxazone base is, however, more readily dissociated and can be extracted from admixture with the oxazine by shaking with xylene, ether, or neutral fat. Accordingly, when a pathological section is stained in a boiled, aqueous solution of Nile blue sulfate, the protein matter is colored blue by the unchanged oxazine. The neutral fat globules, on the other hand, extract the oxazone base from the solution of its salt and are stained red.

It is evident from the foregoing that Nile blue sulfate is water soluble and in aqueous solutions possesses a blue color. When it is converted to the oxazone and the free base extracted, it imparts a red color to neutral fats in which it is soluble.

When a solution of the free oxazone in oil is mixed with a fatty acid, such as oleic acid, the red color is changed to blue. This reaction is specific and forms the basis of the following medium.

Preparation of the Medium

(a) Dissolve 20 g. agar (Bacto) in 1000 cc. of beef infusion broth prepared according to the directions of Zinsser and Bayne-Jones (1935). Filter thru absorbent cotton and adjust the reaction to pH 7.4. Distribute in 100 cc. amounts in flasks and sterilize in the autoclave at 15 pounds pressure for 20 minutes.

(b) *Dye-fat Emulsion:* Dissolve 4 g. ossein gelatin (200 Bloom, isoelectric point at pH 5.6) in 100 cc. of distilled water at 60° C. Add sufficient *N*/10 NaOH solution to raise the pH to 7.2. The gelatin solution, while still warm, is shaken vigorously in a large stoppered bottle with 100 cc. of cotton-seed oil (U.S.P.) to which has been added 0.1 g. Nile blue sulfate (C.I. No. 913, dye content 90%, solubility 0.15 g. in 100 cc. water)[3]. The mixture is then put thru a hand homogenizer until the oil globules are about 10 μ in diameter. This emulsion has a light blue color and after sterilization in the autoclave at 15 pounds pressure for 20 minutes turns deep pink. At room temperature the emulsion soldifies, but by placing it in the incubator at 37° C. a day before it is to be used no difficulty in pipetting is experienced.

(c) *Preparation of Plates:* When plates are to be poured 100 cc. of the sterile infusion agar base are liquified, and while still hot 10 cc. of the sterile dye-fat emulsion are added aseptically. The whole is well mixed and allowed to cool to 45° C. before being poured into plates. Before inoculation the medium has a rich salmon-pink color.

The medium is equally efficient both with pour and streak plates, the former being desirable for quantitative work. Colonies of organisms which decompose fats appear as light blue colonies surrounded by a narrow zone of decolorized, transparent medium when incubated for 3–4 days at room temperature. Non-lipolytic colonies appear white or pink.

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[3]The sample employed by the writer was purchased from Hartman-Leddon Co., Philadelphia, Penna.

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A NEW SHARPENING BACK AND PROCEDURE OF SHARPENING FOR MICROTOME KNIVES

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THE KNIFE BACK

In the course of several years' histological research, the writers have had constant concern with the sharpening of microtome knives. During this time they have devised a new sharpening back (patent pending) and a modification of the usual procedure in hand sharpening which they believe to be of sufficient value to warrant inspection and criticism.

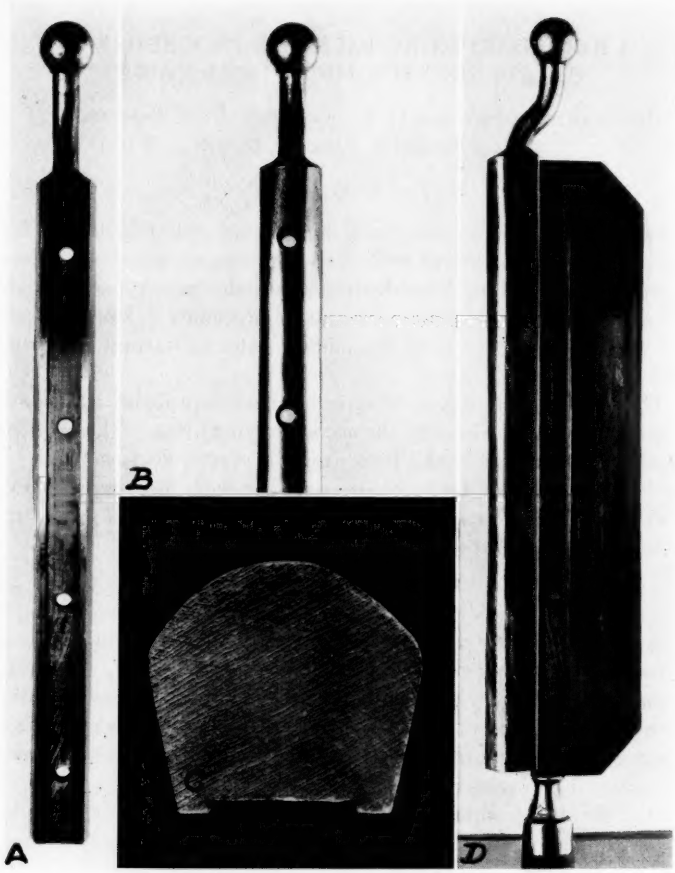
The new detachable knife sharpening back is probably sufficiently illustrated and explained by the accompanying plate. The principal advantages claimed for this back are: it is exactly replaceable in one position; it is immovable in use and has long wearing qualities. Two such sharpening backs have been made and during a year's trial have thoroly fulfilled expectations.

THE SHARPENING PROCEDURE

In describing their sharpening procedure, the writers will, of course, attempt to give only supplements to the general method. They also wish to state that their modification of general sharpening procedure in no way represents their belief that it is the only efficient method. Rather, it is their practical statement of conclusions reached after considerable experimentation.

THE HONE: A combination grit carborundum stone, 8×2×1 inches (No. 108 made by the Carborundum Co., Niagara Falls, New York) has been found most satisfactory. (In the writers' opinion this length of stone is quite sufficient for a 180 mm. knife.) Previous to use, a new stone should have all edges slightly beveled to prevent chipping. In use the stone should rest on two or three thicknesses of wet cardboard which serve to hold the stone in place and provide a slight "give" as a preventative against injury to the knife edge. Distilled water is used as a lubricant in honing. The usual correct treatment of the stone in honing procedure should, of course, be practiced.

It has been found to be a distinct advantage to grind away the heel and toe corners of the cutting edge (shown in D of the accompanying



DETACHABLE SHARPENING BACK FOR MICROTOME KNIVES

(A) The sharpening back with view of channeled side.
(B) Reverse side of (A) showing rounded back and countersinking for attaching screws.
(C) An enlarged cross-section view of the sharpening back. The outline represents the rounded back at top, the grinding faces at the sides, and the channel below.
(D) The assembled sharpening back and knife ready for honing. (Note cutting down of knife blade at heel and toe to aid in honing.)

plate). This greatly facilitates honing at the knife ends and thereby aids in keeping a straight edge. Knives which have been ground down so that the cutting facets are wider than 4 or 5 mm. may (using care) be hollowed out with a power grinder so that the cutting facets

are narrowed to 2 or 3 mm. Slightly varying width of the cutting facets seems to have no ill effect on the knife's cutting qualities if the edge is straight and the angle between the cutting facets is approximately the same at all places along the blade. In preparation for honing, the knife and knife-sharpening back should be carefully cleaned—particularly the back of the knife blade and the channel of the sharpening back so that an exact union is made.

The honing operation: The usual heel to toe diagonal stroke is used and the progress of the grinding on both cutting facets should be observed under a low power microscope. Such examination enables the operator to judge his faults in honing and the completion of grinding. One of the common difficulties in hand honing is the production of a bowed edge. Regular honing at either end of the blade will greatly obviate this. Short strokes of the same diagonal as is used in full length honing should be made with occasional full length strokes to join the grindings smoothly. With correct technic the depression at the blade center may be easily kept within 1 mm. in 150 mm. of knife edge.

First stropping: This is done on a length of 3-inch canvas belting laid over a $2\times3\times15$ inch piece of wood and tacked over at the ends. Tripoli powder wet with water is used as an abrasive. The "honing" stroke is the same as for leather stropping—toe to heel, back foremost. The sharpening back is kept in place and both hands used as in honing. The progress of the polishing is also watched under the low power microscope.

Second stropping: The stropping is completed on leather. Care should be taken to keep the strop taut to prevent excessive "rounding" of the edge. The writers judge that when the knife will clip a human hair at a distance of 1 cm. from the hand the knife is ready for average sectioning purposes.

Acknowledgment is made to the Works Progress Administration for technical assistance under O. P. 465-94-3-39.

LABORATORY HINTS

FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi — *Abstract Editor*

MICROSCOPE AND OTHER APPARATUS

ZDANSKY, O. **An improved apparatus for the serial sectioning of fossils.** *Science*, **88**, 385–6. 1938.

The modifications here suggested apply to Simpson's holder by making it more sturdy and increasing its accuracy. The number of parts is smaller and its design is kept within the range of an average mechanic's skill. It consists essentially of a holder carrying a millimeter scale above the thread. A sleeve rotates around it with divisions on its conical upper end and the 2 scales afford micrometer readings to 0.01 mm. Advantages claimed are: There is no delicate, easily bent pointer; the threads are protected; the scale is an integral part of sleeve; and mistakes in taking readings are less probable.—*J. A. de Tomasi.*

PHOTOMICROGRAPHY

PIJPER, A. **Dark-ground studies of flagellar and somatic agglutination of B. typhosus.** *J. Path. & Bact.*, **47**, 1–17. 1938.

The author finds sunlight superior to artificial light, including a 20 amp. arc lamp, for observation and photography of flagella of living bacteria. The source of sunlight is a Zeiss heliostat mounted on an adjustable glass plate. A collecting lens of 2 meter focus stands between the heliostat mirror and the microscope condenser. The microscope mirror is omitted. Coolers filled with Mohr's salt are indispensable. The other equipment includes a Zeiss cardioid condenser. New slides are used, and are prepared by dipping into a collodion solution. When the skin is nearly dry it is pulled away taking the dirt with it. Oil immersion apochromats 35× and 60× with 20× ocular make a useful combination for photography. Zeiss photographic equipment is used thruout: Contax camera with new "beam-splitter" developed for use with the Zeiss cinema camera.

The bacteria are suspended in a vaseline-sealed wet film preparation, one corner of which is left unsealed to allow diffusion of air.—*S. H. Hutner.*

MICROTECHNIC IN GENERAL

HAGAN, H. R. **Overcoming static when sectioning with the microtome.** *Teaching Biologist*, **8**, 59. 1939.

Trim the mounted block on its upper and lower sides, dip in melted soft paraffin, cool quickly, trim the ends of the block, and section. The soft paraffin will hold the sections together and prevent the ribbon from breaking so easily.—*Virgene W. Kavanagh.*

DYES AND THEIR BIOLOGICAL USES

CH'IN, T. L. **Influence of color filters in photodynamic action of fluorescent dyes on gonococcus.** *Proc. Soc. Exp. Biol. & Med.*, **38**, 697–700. 1938.

Various dilutions (10^{-1} to 10^{-10}) of eosin, methylene blue, trypaflavin, mercurochrome, protargol, argyrol, and dimethyl and tetramethyl paraphenylenediamine hydrochloride were made in dist. water. Suspensions in these dyes were exposed

to light, using Eastman Kodak filters red (F29), orange (G15), yellow (K-1,6) green (B58), blues (H45 and C49) and violet (D35). Samples were tested for viability after 5–120 minutes of exposure. The gonococcus was particularly sensitive, even more so than other Neisseria; control tests indicated survival in dye dilutions not treated with light. Trypaflavin and methylene blue were most effective with an orange filter and eosin with green and light blue filters. Organisms with dilute dyes appeared, in a general sense only, to be sensitive to selected wave lengths of light.—*M. S. Marshall.*

FIGGE, F. H. J. **Inhibition of tyrosinase melanin formation by sodium bezenone-indophenol.** *Proc. Soc. Exp. Biol. & Med.*, **39**, 569–71. 1938.

Sodium bezenone-indophenol prevents the formation of pigment granules, apparently by its action on tyrosinase. The pallor induced in young amphibian larvae by this and similar dyes may be explained on this basis.—*M. S. Marshall.*

GRAFFLIN, A. L. **The absorption of fluorescein from fresh water and salt water by Fundulus heteroclitus, as judged by a study of the kidney with the fluorescence microscope.** *J. Cellular & Comp. Physiol.*, **12**, 167. 1938.

Specimens of the euryhaline species, *Fundulus heteroclitus*, were exposed to a dilute solution of fluorescein in fresh water and sea water. The animals were killed after different periods of immersion, and the extent of absorption of the dye was estimated by a study of the kidney with the fluorescence microscope. Observations indicate that the dye is absorbed quite markedly from sea water but only in traces from fresh water. These results are interpreted in terms of the obligatory swallowing of sea water in marine teleosts, and absence of such swallowing in fresh water teleosts.—*L. Farber.*

IVANOV, M. F. and BRAUN, A. A. **On the problem of permeability of tissue membranes.** *Arch. Russ. Anat. Hist. Embriol.*, **19**, 161–74. 1938.

Striated muscle membranes were found best for studying the permeability of tissue membranes. Neutral red and cyanol were the most suitable dyes. Results obtained with these two dyes of opposite reaction are closely comparable. It is found that permeability of live membranes increases with time. In the case of a dead membrane, however, permeability is consistently lower up to the fifth hour, after which time its value increases faster than in the case of the live membrane. This is probably caused by a greater absorption of the dye by the dead membrane in the first few hours until saturation is reached, thus allowing greater, or faster, permeation. Temperature increases up to 28° C. enhance the permeability of the live membranes.—*J. A. de Tomasi.*

MAURER, F. W. **Absorption of acid indicator dyes by living frog muscle.** *J. Cellular & Comp. Physiol.*, **12**, 379. 1938.

Phenol red absorption by living muscles can be accounted for without involving any absorption by the muscle cells. Killed muscle cells absorbed large amounts of dye. Muscle cells made acid in death absorbed large amounts of dye, while muscle made alkaline in death absorbed little more than living muscles.—*L. Farber.*

SMOILOVSKAYA, E. J. **The supravital staining of surviving sections of normal tissues in physiological and hypotonic surroundings.** *Arch. Russ. Anat. Hist. Embriol.*, **19**, 105–15. 1938.

In this study neutral red is made up in concentrations varying from 1:200,000 to 1:50,000 in Ringer-Locke soln. With sections from such materials as live lung, kidney and liver from the common laboratory animals it is found that concentrations higher than 1:5,000 are toxic. In isotonic solutions, the dye accumulates promptly in the cytoplasm in epithelial cells, while in hypotonic solutions the nuclei are rather apt to take on the dye. This reversal of the reaction appears to be linked to the loss of phosphates from the tissues into the surrounding fluids.—*J. A. de Tomasi.*

STRELING, G. S. **On the physiological gradient. III. Vital stains reduction gradients in asphyxiation in Oligochaeta and their correlation with injuries.** *Arch. Russ. Anat. Hist. Embriol.*, **19,** 226–43. 1938.

Early experiments proved that certain indicators will cause injury to such worms as *Limnodrilus sp.* and *Tubifex tubifex.* This study is intended to find an interpretation of the oxidation-reduction gradients observed, using methylene blue, Janus green, thionin, and brilliant cresyl blue as vital stains and indicators. It is found that prolonged staining during asphyxiation injures the tissues more rapidly and tends to shorten the reduction time of the indicators. Furthermore, axial (total body length) as well as portional (partial lengths), and dorsoventral gradients are observed in the animals under study. Typical observations: the redox potential may be lowered by mechanical injury, by heat, and by the action of Janus green. It appears that the loss in potential can be correlated with an increase in adsorption by the tissue protoplasm, indicating a gain in stainability.—*J. A. de Tomasi.*

TENNENT, D. H. **Some problems in the study of photosensitization.** *Amer. Naturalist*, **72,** 97–109. 1938.

This work was undertaken to study the experimental changes induced in the cell division of eggs of the sea urchin by the combined action of dyes and sunlight. Out of 30 dyes used, results with neutral red, brilliant green, auramine O, acridin, eosin Y and picric acid are reported. In every instance irradiation with sunlight raised the effectiveness of dilute dye solutions to the level of results produced by strong solutions without irradiation. Every dye has its own characteristic effect. Irradiation in dye solution is far more effective when heat is part of the system. Photodynamic action is not proportional to the degree of fluorescence of a dye. The possibility of a photodynamic effect by photo-compounds of the irradiated dyes may be considered, but has not been proven.—*J. A. de Tomasi.*

T'UNG, T. **Intracutaneous immunization of rabbits with photodynamically inactivated Type I pneumococcus.** *Proc. Soc. Exp. Biol. & Med.*, **39,** 159–61. 1938.

Pneumococcus Type I, inactivated by methylene blue and light, was compared with formalin treated suspensions. Photodynamically treated suspensions are less susceptible to autolysis and are antigenically superior as judged by agglutination tests, precipitin tests with soluble specific substance, complement fixation tests and protection tests.—*M. S. Marshall.*

ANIMAL MICROTECHNIC

BLACK, E. **The staining reactions of fats after the use of various dyes and fixing agents.** *J. Lab. & Clin. Med.*, **23,** 1027–36. 1938.

Fat was injected intravenously into a white rat. After asphyxia occurred representative pieces of lung were placed in normal saline for fresh frozen sectioning; other pieces were fixed in various solutions, frozen, and sectioned. The technics of fixing and staining were those described by Warthin in Practical Pathology. Several tables are presented for convenience in identifying various fats fixed with different reagents. The fat dyes used were: Scharlach R, Sudan III, Nile blue sulfate, OsO_4, and indophenol. The author considers poppy seed oil, lipiodol, olive oil, lard, butter fat, mineral oil and paraffin. The fixatives used were formol, $HgCl_2$, and Müller's fluid. Within the limited group studied, vegetable oils stained somewhat more deeply than did animal fats.—*P. R. Beamer.*

COMMONER, B. **On the nature of the staining of unfertilized Chaetopterus eggs by neutral red.** *J. Cellular & Comp. Physiol.*, **12,** 171. 1938.

The uptake of neutral red by unfertilized *Chaetopterus* eggs was found to be a first order reaction, in which the staining rate is proportional to the amount of unstained, stainable material in the eggs. Within limits the staining rate was proportional to the outside neutral red concentration and was increased by a rise in temperature up to 20° C. Above this temperature stained eggs lose their

neutral red binding capacity and set free the bound stain. The rate of loss of neutral red at temperatures up to 43° C. was proportional to the rate of heat denaturation of the egg protein. It was concluded that the staining of the eggs is due to the combination of the stain and some protein constituents of the cells.—*L. Farber.*

COOPER, Z. K. and SCHIFF, A. **Mitotic rhythm in human epidermis.** *Proc. Soc. Exp. Biol. Med.*, **39,** 323–4. 1938.

To count the number of mitotic figures in the epidermis, drop the fresh skin into 1% acetic acid for 24 hr., separate epidermis from dermis and discard dermis, stain epidermis *in toto* in Ehrlich's hematoxylin, dehydrate in alcohol, clear in oil of wintergreen and xylene, mount.—*Virgene W. Kavanagh.*

DEBAISIEUX, P. **Organes scolopidiaux des pattes d'insectes.** *La Cellule*, **47,** 79–202. 1938.

Detachment of sections of chitinous insect parts from the slide during staining may be prevented by adding a temporary collodion film. After removing paraffin with xylene and passing sections thru abs. alcohol, immerse them in a solution of collodion in at least 4 vols. of abs. alcohol+ether; then immerse in 80% alcohol, hydrate and stain. After dehydrating the stained sections with abs. alcohol, dissolve collodion film in ether; then pass sections thru abs. alcohol and xylene and mount in balsam.—*L. W. Sharp.*

DEI POLI, G. and POMERRI, G. **Un metodo semplice e sicuro di colorazione della sostanza cromofila (zolle di Nissl) delle cellule nervose.** *Monit. Zool. Ital.*, **49,** No. 5, 123–4. 1938.

Fix a small piece of nervous tissue (removed as soon as possible after death) for 24 hr. or more in 95–98% alcohol, or in 10% formalin (in water or physiological salt solution). Dehydrate in alcohols, clear in xylene and embed in paraffin. Immerse in the following staining mixture for 3–4 min.: Dist. water, 100 cc.; carbol fuchsin (basic fuchsin, 0.2 g.; conc. phenol soln., 1.0 cc.; 95% alcohol, 2.0 cc., dist. water, 20.0 cc.), 2.5 cc.; glacial acetic acid, 0.5 cc. Wash rapidly in dist. water. Differentiate for 3–5 min. in the following mixture: dist. water 100.0 cc.; formalin, 1.0 cc.; glacial acetic acid, 1.0 cc. Wash sections in dist. water; dehydrate in a series of alcohols; clear in xylene; mount in neutral Canada balsam.

• Sections of tissue fixed in formalin do not need as long a treatment with the formol-acetic acid mixture and should be washed several minutes in 95% alcohol until the surrounding tissue seems to be decolorized.

With this method the staining of the tigroid substance is uniformly good and controlled differentiation under the microscope is not necessary. The chromophile substance and nucleoli are stained sharp red; the nucleus and all other parts of cells and tissue remain colorless.—*A. B. Dawson.*

FRIELING. **Universal-methode für die Färbung von Bindegewebe und elastistischen Fasern.** *Mikrokosmos*, **31,** 82. 1938.

Solution A: Chrom-Hematoxylin "H" 2.00 g., tap water 100.00 ml.; dissolve on water bath 5–10 min., cool and filter. *Solution B:* Blochmann elastin stain "H" 0.5 g., 70% alcohol, 100.00 ml.; filter.

Staining Procedure: Deparaffinize sections; stain in Sol. A. 10–15 min.; rinse in water to remove excess stain; rinse in 70% alcohol; transfer to elastin stain (Sol. B); rinse in 95% alcohol; dehydrate; xylene; balsam.

Result: Nuclei black, collagen fibers blue, elastic fibers dark brown. *Note:* If red nuclei are desired, stain in Kernechtrot-aluminum sulfate for 5 min. after elastin stain. (Kernechtrot-aluminum sulfate "H" 2.00 g., hot water 100.00 ml.) —*J. M. Thuringer.*

HSÜ, F. **Étude cytologique et comparée sur les sensilla des Insectes.** *La Cellule*, **47,** 7–60. 1938.

A solution for injecting larvae of *Choaborus crystallinus* is made as follows: Dissolve 0.2 g. Grübler's methylene blue in 10 cc. dist. water. Add 4 drops of 25% HCl and then 0.4 g. rongalite white. Heat gently until clear. Filter if

necessary and store in stoppered bottle in the dark for a few days. The solution retains its efficacy for 2 weeks. Just before injection mix 1 vol. of the solution with 4 vols. of 0.75% NaCl. Inject 2–4 drops of the mixture into each larva. Fix in sat. soln. of ammonium molybdate.—*L. W. Sharp.*

LEACH, E. H. **A new stain for mucin.** *J. Path. & Bact.*, **47**, 637–9. 1938.

Within a few months, a sat. solution of Sudan black B in 50% diacetin hydrolyzes and loses its property of staining fats, but will stain mucin in both frozen and paraffin sections. The new stain shows greater stability and specificity. It may also be produced by hydrolysis of Sudan black B with acetic acid, forming a new dye "mucisudan".

Prepare the stain by adding 2 g. mucisudan to 100 cc. 0.5% HCl in 70% ethanol. Allow to stand at least two days on a hot plate; filter. A slightly less satisfactory solution is prepared by boiling, cooling, and filtering. Tissue is preferably fixed in Zenkerformalin. Before staining place the sections in acid alcohol 1 min., then in the stain solution 1 hr. Treat with acid alcohol a few minutes; counterstain in carmalum or neutral red. Dehydrate and differentiate in abs. alcohol a few minutes. Mucin, cartilage and occasionally elastic fibers stain dark brown; nuclei, red.

It is difficult to over-differentiate. If the differentiation period is shortened, the granules of mast cells are stained. Alcohol and all other reagents must be free of traces of other dyes. The stain solutions require occasional filtration.

Greatly improved preservation and staining of mucin is obtained if 96% alcohol is used instead of water for spreading. Smear slide with conc. soln. of glycerol and egg albumen. Flatten out sections on 96% alcohol, by warming slide on hot plate. Dry slide at 55° C. at least 12 hr. Remove paraffin with xylene. Treat with abs. alcohol, then with 1.0% celloidin in a mixture of alcohol and ether 1:1. Allow to partly dry. Place in 90% alcohol and stain as usual.—*S. H. Hutner.*

LEACH, E. H. **Fat staining with Sudan black B.** *J. Path. & Bact.*, **47**, 635–7. 1938.

Sudan black B (I. G. Farbenindustrie) in 50% diacetin (glycerol diacetate) stains nuclei red; lipid substances, including myelin, stain blue-black. Tissue should be fixed in 5% formalin in 0.9% saline for 24 hr., or in Zweibaum's fluid, made by mixing equal parts A and B. Sol. A: 3% $K_2Cr_2O_7$, 6 cc.; 2% CrO_3, 3 cc.; dist. water, 5 cc. Sol. B: 2% OsO_4. After fixation wash 24 hr. in running water. Embed in gelatin and cut frozen sections. Transfer 5-10μ sections from dist. water to 50% diacetin for 30 sec., preferably with agitation. Stain 15 min.–2 hr. in sat. soln. Sudan black B in 50% diacetin. The stain should be filtered before use. Staining may be shortened by warming in the paraffin oven without danger of precipitation of the dye. Transfer to 50% diacetin for 30 min., then float on clean water. Mount in Apathy's medium. The solution should be discarded when it starts to smell of acetic acid (caused by hydrolysis of the diacetin).—*S. H. Hutner.*

MONNE, L. **Über experimentell hervorgerufene strukturelle Veränderungen der Golgi-apparates und der Mitochondrien sowie über Bildung von Myelin-figuren in Spermatocyten und Spermatiden von Helix lutescens.** *Protoplasma*, **30**, 582–91. 1938.

In dist. water the mitochondria swell while the Golgi apparatus remains almost unchanged. The swelling of the mitochondria can be followed in a 0.4% hypotonic solution of NaCl containing pyronin. The stain colors only the periphery of the swollen mitochondria. In 0.73% isotonic NH_4Cl and in 0.7% isotonic NaCl containing NH_4OH the mitochondria remain unchanged while the Golgi apparatus undergoes marked structural changes suggesting liquefaction. The Golgi of the spermatids behave similarly.—*R. Chambers.*

MONNE, L. **Über Vitalfärbung des Golgi-apparates und der ergastoplasmatischen Strukturen in einigen Gastropoden-Zellen.** *Protoplasma*, **30**, 460–6. 1938.

A procedure is given for staining epithelial cells of the vas deferens, of the ducts of the receptaculum seminis and of the finger-like and salivary glands of *Helix*

lutescens, H. pomatia and *Tachea austriaca*. Fragments of the organs were placed for about an hour in 0.73% isotonic NH_4Cl, then for 15 min. in 0.8% isotonic NaCl and finally for 10–15 min. in 1.15% isotonic $NaHCO_3$ containing a fairly strong mixture of dahlia and chrysoidin in equal parts. The best results were obtained with the salivary gland cells of *Helix lutescens*. The cells are large and well suited for the investigation.—*R. Chambers.*

NIERO, G. **Sulla nuova tecnica di Bodian per le neurofibrille.** *Monit. Zool. Ital.*, **49**, No. 6, 149–53. 1938.

Method for material containing bony tissue: Fix in Bodian's formol acetic alcohol (formula 2) for 3 days or longer; wash in dist. water for several hours; decalcify completely in 4 to 5% nitric acid. Wash in dist. water for 48 hr. (keep slowly moving with Thoma's apparatus); dehydrate in the alcohol series and embed in paraffin or celloidin; impregnate the mounted sections according to Bodian. In sections of the spinal cord, the nerve cells with neurofibrillae, ganglion cells, and peripheral fibers are clearly demonstrated.

Method for impregnating dissociated intestinal wall: Fix the entire intestine using Bodian's formula 2, preferably for 36–48 hr.; dissociate and wash in dist. water rinsing for 4–5 hr.; immerse in a bath of 1% protargol (Protargol Bayer), containing 5 g. of metallic copper, for 5–6 hr. at 37° C. Reduce as indicated by Bodian. If desired, tone with gold. Mount in balsam or glycerin. With delicate impregnations, the sympathetic plexi as well as fibers and nerve cells can be demonstrated.—*A. B. Dawson.*

NITTIS, S. **The nature and the mechanism of the staining of the erythrocytic reticulum.** *Amer. J. Med. Sci.*, **196**, 177–9. 1938.

Reticulum in immature erythrocytes is a precipitate caused by the interaction between a basophilic substance in the cell and the vital dye. In counterstaining the reticulocytes with Wright's stain, the reticulum stain is replaced by methylene blue.—*J. A. Kennedy.*

RASMUSSEN, A. T. **The innervation of the hypophysis.** *Endocrinology*, **23**, 263, 1938.

A method is given for the staining of the hypophysis to bring out the nerve fibers more uniformly. The procedure is as follows: Fix small pieces in abs. alcohol containing 6 drops of NH_4OH per 100 ml., 2–3 days; wash in running tap water to remove ammonia, 12 hr.; treat with 3% nitric acid, 2 hr.; rinse in tap water, a few minutes to an hour depending on the size of the piece of tissue. Treat with several changes of 80% alcohol, a few hours; 80% alcohol+4 drops NH_4OH per 100 ml., over night; pure pyridine, 12 hr. Wash out pyridine with repeated changes of dist. water until no odor of pyridine is detectable. Treat with 2% $AgNO_3$ in the dark, 24 hr.; 0.75% $AgNO_3$ in the dark, 24 hr.; 2% $AgNO_3$ in the dark, 24 hr. Rinse quickly in dist. water; reduce in 4% pyrogallic acid, 24 hr.; dehydrate and clear (details not given); embed in medium paraffin so that sections can be cut at least 10 μ thick.—*L. Farber.*

ROBB-SMITH, A. H. T. **Reticulosis and reticulosarcoma: a histological classification.** *J. Path. & Bact.*, **47**, 457–80. 1938.

Lymph nodes removed at biopsy for histological examination should be fixed intact in 4% saline formalin for about 6 hr., then bisect the node and allow to fix thoroly. This avoids distortion due to capsular contraction if the node is bisected unfixed. Additional material may be fixed in other fluids.

Paraffin sections are stained with hematoxylin and azo-eosin, iron hematoxylin and Van Gieson stain (1%), and aq. toluidine blue to demonstrate basophil cytoplasmic granules.—*S. H. Hutner.*

ULPTS, R. G. E. **Morphologische Studien zur Physiologie der Erythrocyten und zur Blutplättchengenese.** *Folia Haematol.*, **60**, 205–21. 1938.

The technic permits the demonstration of the following constituents. 1. An outer zone containing hemoglobin. 2. An inner lyophilic hyaline body. 3. Fre-

quently observed paired granules. 4. A body which upon release into the blood stream simulates a blood platelet. 5. A "restbody". Guinea pigs furnish the most suitable erythrocytes.

Check blood by ear puncture and examination of 10 stained smears. Inject 0.5 ml. phenylhydrazin soln. (0.75 ml. phenylhydrazin base to 25 ml. dist. water). Make 10 additional blood smears at intervals of 24 hr. Fix a portion in methanol and stain 30 min. in Giemsa. Fix the other portion in Dominici's fixative (aq. sat. soln. $HgCl_2$ and tinct. iodine, 20:2; shake well, filter, and add 2 parts of formalin). The presence of an erythroblast in a smear indicates the proper time for proceeding. Injection may be repeated in 72 hr. if no erythroblasts appear. *Procedure:* Anesthetize the animal. Remove the sternum high enough to expose the trachea and heart. The assistant cuts all the cervical vessels in one operation, simultaneously the operator plunges the 5 ml. syringe full of Dominici's into the left ventricle, and empties the contents into a porcelain dish with 1000 ml. of normal saline and transfers to a tall vessel to permit settling. The supernatant fluid is drawn off and replaced with dist. water. Invert the vessel twice to remove excess fixative. After settling out, draw off again. From the brownish material prepare smears. The air dried preparations take a Giemsa or a silver impregnation stain. Prepare smears of the rapidly fixed erythrocytes adhering to the endocardium by touching coverslip to the open heart. Alkaline Giemsa for 24 hr. and silver impregnation by Gordon's method gave best results.—*J. M. Thuringer.*

PLANT MICROTECHNIC

BELL, H. P. and FACY, V. **Microtechnic for winter buds.** *Canadian J. Res.*, Sec. C. **15,** 129–34. 1937.

The authors give rather detailed procedures for preparing winter flower bud material for microscopic examination, including directions for embedding. Alcoholic stains were used to help in fixing sections onto the slide. Safranin in 50% alcohol was used for nuclei and acid fuchsin in 70% alcohol for a general stain.—*G. I. Wallace.*

HUTCHINS, H. L. and LUTMAN, B. F. **Spine development on the spores of Ustilago zeae.** *Phytopathology,* **28,** 859–60. 1938.

The growth of a spiny wall from a gelatinous matrix during development of the smut spores has been a controversial point for some time. The following technic, used for staining the sections of corn bolls infected with the mycelium of *Ustilago zeae,* differs markedly from the Hutchinson stain (*Phytopathology,* **26,** 293–4, 1936) from which it was derived.

Fix material in Allen-Bouin mixture, cut and transfer sections to water. Stain 24 hr. in sat. orseillin soln. (1 g. of dye in 20 cc. 3% acetic acid). Wash quickly with 50% alcohol to remove excess stain. Stain 24 hr. in sat. anilin blue soln. (1 g. of dye in 100 cc. 3% acetic acid). Dehydrate with abs. alcohol, xylene, and mount in balsam. A permanent differential stain is obtained in which the exospore stains red, and later the red spines protrude into the blue layer of the primitive wall.—*J. A. de Tomasi.*

MÜNCHBERG, P. **Ein auch für den Mikroskopier wertvolles Verfahren zur Konservierung von grünen Pflanzen und deren Teile.** *Mikrokosmos,* **32,** 14–5. 1938.

The method of v. Pillich and Winkler, developed primarily for the preservation of botanical materials in their natural colors, was found suitable for histological investigation after rinsing the material in water and dehydrating in alcohol.

Place material in large vessel containing sufficient 0.5% aq. zinc sulfocarbolate solution to cover specimens and exhaust the air for 20–30 min. Tap water may be used because it does not react with Zn ions to form precipitate. The material is now transferred to a tall cylindrical vessel half filled with a solution containing 1% zinc sulfocarbolate and 2% sodium benzoate. The filling of the vessel is now completed with a solution of 2% Na_2SO_3 and sodium benzoate (meaning 2% of each solution?). The fine precipitate formed is siphoned off in a week and the material transferred to 10% HCl in which the adhering precipitate is removed. After washing the material in water it is returned to the filtered solution in the

tall vessel. Zinc sulfocarbolate was selected because it was found to form a leuco-compound with chlorophil, while the sodium benzoate merely acts as a preservative and bactericide. The Na_2SO_3 neutralizes the zinc sulfocarbolate (after hydrolyzing).—*J. M. Thuringer.*

WOLF, F. T. **Cytological observations on gametogenesis and fertilization in Achlya flagellata.** *Mycologia*, **30**, 456–67. 1938.

Cultures of this fungus were maintained on boiled hemp seed in dist. water. Sexual organs were produced in abundance. For fixation, best results were obtained with mixtures of 0.3% chromic acid and 0.7% glacial acetic acid, diluted with dist. water until plasmolysis was reduced to a minimum. Embed in paraffin, cut sections 5 μ or 10 μ in thickness, remove paraffin in xylene, and pass slides down graded series of alcohols to water. Treat slides as follows: K-KI 20 hr. (no conc. given), 5 min.; wash in water a few seconds; crystal violet sol. (no conc. given), 30 sec. to 5 min.; wash in water; K-KI soln. (no conc. given), a few seconds; wash in water; flood slide with 95% alcohol and drain; flood slide with sat. picric acid in 95% alcohol and drain; flood slide with abs. alcohol; destain further in clove oil until desired color intensity is secured; clear in xylene; mount in balsam.—*Elbert C. Cole.*

WOODROFF, J. G. **Method for "fixing ice crystal patterns" in frozen products.** *Science*, **89**, 87. 1939.

It is difficult to obtain good paraffin sections of fruits and vegetables preserved by freezing. The tissues are very delicate and easily distorted. The author recommends the use of a freezing microtome, cutting at an average temperature of 18–20° F. Materials, microtome and supplies are chilled for 1 hr. before use. Where fine ice crystallization is produced, sections can be cut as thin as from 10–30 μ.

Kill and fix by floating sections in the following chilled formic-acetic-alcohol soln.: acetic acid, 5%; formalin, 10%; alcohol, 70%; water, 15%. This makes the material sufficiently stiff to prevent sticking together, to facilitate handling, and to assure satisfactory ice crystal patterns. The unmounted sections are best stained with eosin, orange G, and basic fuchsin for cell contents; with light green, basic fuchsin, and methylene blue for cell walls. Safranin with Delafield's hematoxylin gives best results for anatomical study of asparagus tips; and orange G, light green, or safranin-light-green for starch grains and cell walls of pears, beans and corn.—*J. A. de Tomasi.*

MICROÖRGANISMS

BISSET, K. A. **The structure of "rough" and "smooth" colonies.** *J. Path. & Bact.*, **47**, 223–9. 1938.

The morphology of bacterial colonies may be studied by means of impression preparations. Inoculate agar plates (blood agar is especially suitable) with a glass rod to avoid scratching the surface. When the colonies are 6–48 hr. old and the surface of the medium perfectly dry, press the agar surface against a cover slip. Fix by immersion in Bouin's fluid until the medium is bleached thruout (usually 2 hr.). Peel the medium away from the cover slip, leaving the colonies attached to the cover slip. Remove the fixative by soaking a few hours in water. The preparation is then stained as usual; dilute fuchsin is useful for those to be photographed.—*S. H. Hutner.*

CHAMBERS, S. O. and SCHOLTZ, J. R. **Clinical application of a stain for spirochaetes (Krajian).** *Arch. Dermat. & Syphilol*, **38**, 217–24. 1938.

The clinical use of the Krajian stain for spirochaetes has been found accurate for identifying *S. pallida* in cases of suspected primary syphilis. It has certain advantages which suggest a wider applicability than the dark-field procedure.—*J. A. Kennedy.*

COPE, E. J. and KASPER, J. A. **Cultural methods for the detection of typhoid carriers.** *Amer. J. Pub. Health*, **28,** 1065–8. 1938.

A comparison was made of the merits of a modified Wilson and Blair bismuth sulfite agar with Endo agar. The modified Wilson and Blair medium consists of: peptone, 1.0%; beef extract, 0.5%; dextrose, 0.5%; Na_2HPO_4, 0.1%; $FeSO_4$ 0.03%; $Bi_2(SO_3)_3$ indicator, 0.8%; brilliant green, 0.0025%; agar, 2.0%. A total of 2,273 examinations of feces and urine were made. In the finding of 452 positive cultures, the $Bi_2(SO_3)_3$ agar, when poured over 5 cc. of a heavy fecal emulsion, accounted for an increase of about 40% of the positive cultures. Endo agar accounted for the recovery of typhoid and paratyphoid bacilli from 23% of the number of positive specimens when $Bi_2(SO_3)_3$ failed. In view of the positive findings with Endo alone, Endo should not be discontinued, but should be supplemented by Bi agar for routine examination.—*M. W. Jennison.*

FITE, G. L. **The staining of acid-fast bacilli in paraffin sections.** *Amer. J. Path.*, **14,** 491–507. 1938.

Tissue should be fixed in alcohol, an alc. solution of formaldehyde, or 5% alc. $HgCl_2$. Mercury deposits are best removed by I_2 solution followed by alcohol and finally $Na_2S_2O_3$. After fixation in $K_2Cr_2O_7$, thoro washing of the tissue block and subsequent treatment of the section with $KMnO_4$ and oxalic acid is recommended. Acid-fast bacilli will not stain after prolonged fixation in either dichromate or formaldehyde solutions or passage thru decalcifying fluids.

Staining solution: New fuchsin (source of dye not given) 1 g., phenol crystals, 5 g., methyl alcohol, 10 cc., dist. water to make 100 cc. Dissolve the dye in the mixture of phenol and alcohol and add the water slowly. The staining solution keeps if protected from evaporation, but deteriorates rapidly in Coplin jars.

Staining time after alcohol fixation: At 20° C., 2–8 hr.; 37°, 1–4 hr.; 60°, 0.5–2 hr.; 90° (steaming), 5 min. After other fixation, 3 to 4 times longer except steaming, 5 min. Prolonged staining at room temperature is preferred. Differentiate with 1% HCl in 70–95% alcohol. Counterstain with methylene blue or hematoxylin if desired.—*H. A. Davenport.*

GUNSALUS, I. C. and STARK, C. N. **Formate ricinoleate and brilliant green bile broths to detect coliform organisms in milk.** *Amer. J. Pub. Health*, **28,** 832–4. 1938.

Formate ricinoleate broth was used to determine the presence of coliform organisms in 655 samples of pasteurized milk. Ninety-eight per cent of the positive presumptive tests confirmed; in the other 2% (3 samples) slow lactose fermenting or related organisms were found. In comparative tests on 221 samples of pasteurized milk, formate ricinoleate and brilliant green, 2% bile broth gave approximately the same number of positive presumptive tests. Brilliant green gave a slightly higher yield of positive presumptives, but some of these were shown to be due to anaerobic spore-formers.—*M. W. Jennison.*

HAGEMANN, P. K. H. **Fluoreszenzfärbung von Tuberkelbakterien mit Auramin.** *Münch. Med. Woch.*, **85,** 1066. 1938.

A procedure is given for the direct detection of tubercle bacilli in sputum, pus, feces, urine, etc., with the aid of the fluorescence microscope developed by the author (*Zentbl. Bakt., I Orig.* **140,** 184. 1937). The rather thin flame-fixed smears are stained as follows: 1:1000 aq. auramin "Bayer" containing 5% "phenolum liquefactum", 15 min.; wash well with tap water, decolorize with an acid alcohol solution (ethanol, 1000 cc.; conc. HCl, 4 cc.; NaCl, 4 g.), 3 min., renewing the alcoholic-HCl solution after 1½ min.; wash well with tap water. Examine the smear without cover glass with the fluorescence microscope, using 180× magnification, apochromatic dry objective and a 3× compensation ocular. For the absorption of the visible and red rays a 3.5 mm. thick "Uvet" lens and a 2% $CuSO_4$ solution are used.

The tubercle bacilli appear as golden yellow fluorescent rods, clearly differentiated from the violet fluorescent background. Eighty per cent agreement with cultural and animal inoculation methods is found. The sensitivity of the method may be increased by treating the material to be tested with antiformin or other enrichment agents.—*L. Farber.*

HOFFMANN, E. **Einfache Dauerfärbung von Hautpilzen und Mikroorganismen mit Azureosinglycerin.** *Klin. Woch.*, **17**, 1622–3. 1938.

A mixture of 2–3 parts of Azure-eosin (Giemsa) and 3–10 parts glycerin is prepared and a drop is placed on a slide with a few skin scrapings, cleared in 10–20% KOH before mounting. This stains the mycelia and spore masses of the fungi dark blue and the cornified epithelium rose. A piece of dead skin may be fastened to the slide with albumin fixative and stained in the same way. Tissues fixed in alcohol and sectioned, or air-dried smears fixed in 70% alcohol, may be stained over night in dilute solutions of Giemsa-glycerin, dehydrated in acetone, cleared in xylene, and mounted in cedar oil. The fungus then stains red, and the finer structures show very clearly. Other organisms, including pneumococcus, may be stained; other tissues also stain satisfactorily.—*Virgene W. Kavanagh.*

ISADA, M. **Geisselfärbung aus alter Bakterienkultur.** *Zentbl. Bakt., I Abt. Orig.*, **142**, 480–3. 1938.

Flagella stains of cultures several days old were found superior to those of young cultures. Six anaerobes were incubated at 37°C. and held for 2–3 days at room or refrigerator temperature. Smears were made from the slants (not the condensation water) using a drop of physiological saline or dist. water on perfectly clean cover glasses which had been dried several hours in a drying oven. The formula used was that of Toda (*Nippon Izi Sinpo* **283**, 113, 1928). *Sol. A:* 20% aq. tannic acid, 5% aq. phenol, sat. aq. alum, 2:5:2. *Sol. B:* sat. alc. soln. of fuchsin or gentian violet, Victoria blue 4R, etc. Smears were stained 0.5–2.5 min. in a freshly mixed, refiltered mixture of Sol. A and Sol. B., 9:1.—*J. P. Sell.*

KRAJIAN, A. A. **A reliable method of staining Spirochaeta pallida in smears.** *Arch. Dermat. & Syphilol.*, **38**, 427–8. 1938.

A swab is moistened in alcohol and the lesion roughly rubbed until it bleeds. Several minutes after all the bleeding stops, a clear serous exudate will appear on the abraded surface; from this smears are prepared and dried in air. Each smear is flooded for 5 min. with a warm No. 1 solution [$UO_2(NO_3)_2$, 1 g.; formic acid (85%), 3 cc.; glycerin (C. P.), 5 cc.; acetone, 10 cc., and alcohol (95%), 10 cc.] washed in dist. water and treated with a weak solution of gum mastic (3 drops of sat. alcoholic solution of gum mastic mixed with 7 cc. of 95% alcohol) for 2 min. The gum is poured off and the breath blown over the surface of the smear, which is then rinsed in dist. water. The slide is set on a metal stand or tripod, flooded with a 1% aqueous solution of $AgNO_3$, heated over a burner until bubbles begin to form (not boiled) and kept at that temperature for 3 minutes. The silvering process is repeated once, the silver poured off without washing, a thin coat of developing solution applied and the slide left under electric light for 2 minutes, being warmed gently with a flame. The developing solution consists of hydroquinone, 0.31 g.; Na_2SO_3, 0.06 g.; 40% neutral, solution of formaldehyde, 2.5 cc.; pyridine, 2.5 cc.; sat. solution of gum mastic in 95% alcohol, 2.5 cc., and dist. water, 15.0 cc. The slide is then washed with dist. water, dried on filter paper and examined under an oil immersion lens. For permanent mounts the preparation may be dehydrated with abs. alcohol, blotted, cleared in xylene and mounted in dammar. Stained smears should be brown. No. 1 solution is stable and keeps for long periods. Ag solution should be prepared from a 10% stock solution each time. Dilute gum mastic should be prepared fresh. The developing solution keeps well for from 2–3 weeks in a light room, after which it deteriorates, the gum mastic separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared. It is important to use fresh developing solution; otherwise the organisms will fail to stain.—*J. A. Kennedy.*

McAULIFFE, H. D. and FARRELL, M. A. **Dye concentration in culture media employed for the analysis of Escherichia-Aerobacter members in milk.** *Amer. J. Pub. Health*, **28**, 1217–21. 1938.

Milk adsorbs a considerable portion of the dye from fuchsin lactose broth and from brilliant green lactose 2% bile broth, thereby lessening the selective action of these media. To obtain enough free dye so that they will be selective for coliform organisms, the content of basic fuchsin in fuchsin lactose broth should be increased from 1 part in 66,666 to 1 part in 9,500, and the content of brilliant

green in the bile medium should be increased from 1 part in 75,188 to 1 part in 30,000. The above dye concentrations in each case are, first, the concentration specified by Standard Methods of Water Analysis for water, and second, the authors' findings for the concentration in milk to give the equivalent of *free* dye specified for water. Studies with different milks are included.—*M. W. Jennison.*

RACICOT, H. N., SAVILLE, D. B. O. and CONNERS, I. L. **Bacterial wilt and rot of potatoes—some suggestions for its detection, verification and control.** *Amer. Potato J.*, **15**, 312–8. 1938.

In connection with a procedure for making smears from vines and tubers, the following modification of the Gram stain is suggested. It is reported to have been devised by G. B. Reed but not to have been published previously.

Prepare the following solutions: (1) 0.25% aq. crystal violet; (2) 1.25% aq. $NaHCO_3$; (3) 2% iodine in a mixture of 1 vol. M/1 NaOH to 9 vols. water (the water added after the iodine has been dissolved in the NaOH); (4) 4 vols. 95% ethyl alcohol in 1 vol. acetone; (5) 1 vol. sat. solution basic fuchsin in 95% alcohol to 9 vols. water.

Flood smear with equal parts of soln. 1 and 2 for about 10 sec., then drain off excess; flood with soln. 3 for about 10 sec. and wash in water; apply soln. 4 until no more color comes away (about 5–10 sec.) and wash in water; flood with soln. 5 for not over 2 or 3 sec., wash and dry.—*H. J. Conn.*

SANDIFORD, B. R. **A new contrast stain for gonococci and meningococci in smears.** *Brit. Med. J.*, **1**, 1155. 1938.

A Gram stain with Pappenheim's stain for counterstain is based on the methods of Fauth and of Scudder.

Stain: Crystal violet, 1 g.; 98% alcohol, 20 cc.; and 1% aq. ammonium oxalate, 30 cc. Leave on smear, 30 sec.; wash with triple strength Lugol's, 30 sec.; blot; decolorize with acetone, 3–4 sec.; wash; counterstain, 2 min.; flood with water (do not wash) and blot.

Counterstain: Malachite green (source not given), 0.05 g.; pyronin (Grübler), 0.15 g.; and dist. water, 100 cc. Cells and nuclei are stained bluish-green; Gram-positive organisms, purple-black; and Neisserieae, red.—*Sara A. Scudder.*

Standard Methods for the Examination of Dairy Products. *7th Ed. Amer. J. Pub. Health*, **28**, 1447–8. 1938.

The forthcoming edition of what was Standard Methods of Milk Analysis has been enlarged to include certain milk products. Bacteriological Methods has been entirely rewritten, with important changes in composition of the agar for the standard agar plate count technic. Methylene blue-thiocyanate tablets, having been standardized, are accepted as standard. Brilliant green lactose bile broth, sodium formate ricinoleate broth, violet red bile agar, and sodium desoxycholate agar are approved for use in detecting coliform bacteria in dairy products. Several changes in methods and new tentative methods for the detection of certain pathogens in milk are given. This new edition will be in print early in 1939.—*M. W. Jennison.*

STARKEY, R. L. **A study of spore formation and other morphological characteristics in Vibrio desulfuricans.** *Arch. Mikrob.*, **9**, 268–304. 1938.

Dorner's method of staining spores was modified as follows: Flood the fixed smear with 5% CrO_3 and heat 30 sec. over boiling water. Wash, flood with carbol fuchsin and heat 10 min. over boiling water. Wash off excess stain, dry and spread over the smear a thin film of nigrosin. Spores are stained a deep red in colorless cells which show distinctly against a gray background. Early stages of spore formation can apparently be detected by this method.—*Merritt N. Pope.*

STEWART, H. M. **The staining of Trichomonas riedmüller, with Wright's blood stain.** *J. Parasitology*, **5**, 473–4. 1938.

Place material on a glass slide and fix with OsO_4 fumes by inverting it over a 5 ml. beaker, in the bottom of which has been placed a piece of folded filter paper

(Whatman 5.5 cm., No. 5). Immediately before use, place on the filter paper 1 or 2 drops of 2% OsO_4 soln. in 1% CrO_3 soln. Expose the Trichomonas to the osmic acid fumes until microscopic examination shows no motility among the organisms (10–30 sec. is sufficient). Air the smear . Cover smear with 5 drops of Wright's stain. Add 15 drops of Sφrensen's buffered soln. pH 7.0–7.6. The solution is mixed by tilting the slide gently 2 or 3 times. Allow the diluted stain to stand 2 min. It is then "floated off". Wash the slide thoroly with the Sφrensen's soln. previously used as the stain diluent. Air dry the preparation.

The anterior flagella and those bordering the undulating membrane stain pink; cytoplasm, blue; posterior part of the axostyle, pink; that portion extending thru the cytoplasm, blue; blepharoplasts, chromatin ring around the posterior portion of the axostyle, endoaxostylar granules and nucleus, dark purple. The cystostome appears as a clear area at the anterior extremity of the body of the organism.—*Elizabeth Bachelis.*